Fibrogenesis, novel lessons from animal models

[1,2]Ellen De Langhe, [1,2]Rik Lories

[1] Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Center, Department of Development and Regeneration, KU Leuven, Herestraat 49, 3000 Leuven, Belgium.
[2] Division of Rheumatology, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium.

Corresponding author: Prof. Dr. Rik Lories, rik.lories@uz.kuleuven.be. Telephone number: +3216342541. Fax number: +3216342543

Abstract

Systemic sclerosis (SSc) is a devastating chronic autoimmune connective tissue disease characterized by vasculopathy, autoimmunity with inflammation and progressive fibrogenesis. The current paradigm of the pathogenesis of SSc is that of an unknown initial trigger, leading to a complex interaction of immune cells, endothelial cells and fibroblasts, producing cytokines, growth and angiogenic factors, resulting in uncontrolled and persistent tissue fibrogenesis by an altered mesenchymal cell compartment. Animal models are of utmost importance to investigate the different steps in the pathogenesis. This review will elaborate on recent findings in established and more recently developed animal models, presenting data on compounds that are in or ready to be translated into clinical trials, or provide interesting new findings in the understanding of the pathophysiology of SSc. We focus on recent findings concerning the vessel – extracellular matrix interaction, the initial triggering aggressor, the concept of autoimmunity and inflammatory changes, the effector cells and their origins and the complex interaction of the different signaling pathways in fibrogenesis.

.

Keywords

Systemic sclerosis, fibrosis, animal model, fibrogenesis

Introduction

Systemic sclerosis (SSc) is a severe, chronic connective tissue disease, characterized by vasculopathy, inflammation with features of autoimmunity and progressive fibrosis (1). A deregulated fibroblast compartment produces and deposits increased amounts of extracellular matrix (ECM), resulting in progressive fibrosis of skin and internal organs, leading to tissue damage and organ failure. SSc remains a challenging disease with a highly variable clinical course and lack of effective therapy options, making it one of the top research priorities in the field of systemic rheumatic diseases.

Fibrosis is the hallmark feature of SSc. The imbalance in deposition and degradation of the ECM components is often considered the ominous result of an initial attempt for regeneration or wound healing that lacks successful confinement and termination of the process (2). The current paradigm of the pathogenesis of SSc is a multistep process where an unknown initial trigger leads to a complex interaction of immune cells, endothelial cells and fibroblasts, with production of cytokines, growth and angiogenic factors, resulting in uncontrolled and persistent tissue fibrogenesis by a modified mesenchymal cell compartment.

The ideal animal model for human SSc should therefore encompass and integrate all features of the disease including vasculopathy, autoimmunity with production of autoantibodies and progressive fibrosis. However, this phenocopy is yet to be described. Nevertheless, several murine models are available and can be used to study aspects of the human disease. This review will highlight a selection of recent findings in established and more recently developed animal models, that either present data on compounds that are in or ready to be translated into clinical trials, or provide interesting new findings in the understanding of the pathophysiology of SSc.

A focus on vessel – extracellular matrix interaction

Microangiopathy with endothelial damage and perivascular inflammation is an early phenomenon in SSc patients (3, 4). The current paradigm suggests that the resulting uncontrolled repair processes culminate in intimal proliferation and adventitial fibrosis of the vessel, with subsequent luminal obliteration causing tissue hypoxia, ischemia and potentially necrosis (5). It is currently unclear whether the initial vessel injury is a toxic, metabolic or autoimmune event and the primacy of vascular injury or autoimmunity in the disease has not been established. New animal models show both vasculopathy and skin and/or lung fibrosis, and are therefore promising tools to study the complex cell and tissue crosstalk that characterizes onset and progression of disease. A series of genetic mouse models has identified potential key players in these processes that fit into a rapidly developing network of cell-matrix interactions, that have been validated in SSc patient tissues and appear strongly regulated by epigenetic factors in the patient population.

Fra-2 transgenic mice

Fra-2 is a signaling molecule from the Fos protein family. Fos proteins (c-Fos, FosB, Fra-1 and Fra-2) form the transcription factor complex AP-1 together with Jun proteins (c-Jun, JunB and JunD). AP-1 activity controls different cell stress responses including inflammation, proliferation, apoptosis, carcinogenesis and wound healing. The serendipitous observation that Fra-2 transgenic mice develop fibrosis, primarily in the lung but also in other organs, sparked interest in its role in SSc (6). Effectively, Fra-2 positive endothelial and vessel-associated smooth muscle cells were found in the affected skin of SSc patients and clearly increased compared to healthy skin (7).

The Fra-2 transgenic mice, in which the murine Fra-2 gene is expressed under the control of the ubiquitous major histocompatibility complex class I antigen H2Kb promoter are viable and fertile but die at the age of 17 weeks in respiratory distress. Increased collagen deposition is observed in the lungs, skin, thymus, heart and gastrointestinal system. At the age of 12 weeks, obliteration and neo-intima formation with perivascular inflammation are observed in pulmonary arteries. These vascular changes precede the fibrotic changes by 2-3 weeks, the latter characterized by interstitial lymphocytic inflammation with excessive collagen deposition and the appearance of structures resembling fibroblast foci and peripheral honeycombing changes. These findings positioned the Fra-2 transgenic mice as a model for SSc-associated pulmonary hypertension and fibrosis (8). In the skin, vasculopathy with perivascular infiltrates and endothelial apoptosis results in a progressive decrease in dermal capillary density further evolving into fibrosis. Notably, there was no evidence for proliferative vasculopathy in the skin (7). Fra-2 transgenic mice also show cardiac changes with endothelial cell apoptosis, reduced capillary density, perivascular

inflammation, myofibroblast differentiation, and myocardial accumulation of collagen, comparable to the changes observed in SSc patients (9). Further analysis of the pulmonary vascular changes in Fra-2 transgenic mice showed a link between arterial remodeling, increased vessel wall thickness, occlusion of pulmonary arteries and increased vascular expression of platelet-derived growth factor (PDGF)-BB and its receptor PDGF-Receptor-b. Of translational interest, pulmonary arterial remodeling and fibrosis development were successfully inhibited in this model by inhibition of the PDGF receptor using tyrosine kinase antagonist nilotinib (8).

The effects of Fra-2 are mainly due to abnormalities in the endothelial and mesenchymal cells: hematopoietic cells do not appear to contribute to lung fibrosis development in this model, as no pulmonary fibrosis is observed in wild type mice reconstituted with Fra-2 transgenic bone marrow. Pulmonary fibrosis also develops to equal extent in Fra-2^{tg}/$Rag2^{-/-}$ mice, indicating that the development of lung fibrosis in this model does not require functional B- or T-cells. The fibrotic response is characterized by an increased number of myofibroblasts with evidence for epithelial-to-mesenchymal transition (EMT) and enhanced expression of profibrotic cytokines (interleukin (IL)-2, IL-4 and IL-6). Of note, no SSc-specific autoantibodies are detected (6). How Fra-2 gets activated in SSc patients is currently unclear but there is evidence that its expression is affected by epigenetic mechanisms. Inhibition of histone 3 lysine 27 (H3K27) histone trimethylation induces the expression of Fra-2, resulting is "spontaneous" skin fibrosis and exacerbated fibrosis when induced by bleomycin or by overexpression of a constitutively active transforming growth factor β receptor type I ($T\beta RI^{CA}$) (10). In conclusion, Fra-2 transgenic mice develop dermal and pulmonary vasculopathy preceding the onset of skin and lung fibrosis, and cardiac changes closely resembling human SSc. Fra-2 might therefore be an interesting novel candidate for molecular-targeted therapies for SSc.

Urokinase-type plasminogen activator receptor ($uPAR)^{-/-}$ mice

The fibrinolytic system consists of serine proteases that play a crucial role in ECM degradation. uPAR is key component in this process as it concentrates the enzymatic activity of its ligand uPA (urokinase) at the cell-matrix interface. There is evidence that uPAR is implicated in the pathogenesis of SSc. A single nucleotide polymorphism located in the promoter region of the uPAR (CD87) gene is a risk factor for SSc vasculopathy (digital ulceration and pulmonary arterial hypertension (PAH)) (11). Levels of uPAR are lower in SSc skin compared to healthy controls. Mice with targeted deletion of the uPAR gene were generated, resulting in complete uPAR deficiency (12). From 12-24 weeks of age onwards, profibrotic cytokines are upregulated (transforming growth factor β (TGFβ), connective tissue growth factor (CTGF), endothelin-1 (ET-1)), resulting in increased dermal thickness with perivascular fibrosis and lipoatrophy. Inflammatory infiltrates are observed as clustered degranulating mast cells in the deeper dermal layers, most prominent in 12-week old mice. Dermal vasculopathy is evident with decreased microvascular density and higher number of apoptotic endothelial cells. No intimal proliferation is observed. Between 12 and 24 weeks of age, the lungs of $uPAR^{-/-}$ mice show progressive cellular infiltration and collagen deposition. However, no evidence for pulmonary angiopathy is detected. So $uPAR^{-/-}$ mice are presented as a murine SSc model with peripheral small vessel vasculopathy and progressive skin and lung fibrosis (13).

Fli1 mutant mice

Fli1 is a transcription factor that plays a crucial role in hematopoiesis and megakaryocyt formation (14). In healthy human skin Fli1 protein is expressed in fibroblasts and endothelial cells and functions as a transcriptional repressor of collagen 1a2 (Col1a2) expression (15). In affected skin of SSc patients, Fli1 expression is reduced in endothelial cells (16) and epigenetically repressed in fibroblasts (17). It was demonstrated that TGFβ acetylates Fli1 in dermal fibroblasts, leading to the dissociation of Fli1 from the collagen and CTGF promoter associated with up-regulation of Col1a1 and Col1a2 genes and down-regulation of the matrix metalloproteinase 1 (MMP1) gene (18). $Fli1^{-/-}$ mice are embryonically lethal and die *in utero* at E11 due to cranial and spinal hemorrhage (19).
The C-terminal transcriptional activator (CTA) domain of the Fli1 gene can function either as a transcriptional activator or repressor (20). To further study the role of Fli1 in collagen expression, mice with a targeted deletion of the CTA domain were generated (Fli1ΔCTA) (21). The skin of Fli1ΔCTA mice had a normal dermal thickness, but had abnormal collagen fibrils with an abnormal wide range of fibril diameter and enhanced Col1a2 expression (22). Albeit that the skin phenotype of these mice is clearly

different from that of SSc patients, the described abnormalities in this mouse model demonstrate the critical role of this gene in homeostasis of the skin. The reduced levels of Fli1 in SSc endothelial cells stimulated further research into the potential role of Fli1 in SSc vasculopathy. For this purpose, mice with a conditional deletion of Fli1 in endothelial cells (Fli1 CKO) were generated. Mice with Cre recombinase under the control of the endothelium specific Tie2 (Tek) receptor promoter were crossed with $Fli1^{flox/flox}$ mice. Fli1 CKO showed a disorganized dermal vascular network with compromised vessel integrity including micro-aneurysmata and dilated capillaries, and markedly increased vessel permeability, recapitulating some aspects of the pathological SSc microvasculature. Expression levels of VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM1), type IV collagen, MMP9, PDGF-B, and S1P(1) receptor were regulated by Fli1, pointing at a role of Fli1 as a regulator of vessel maturation and stabilization. It is therefore hypothesized that reduced levels of Fli1 in endothelial cells play a critical role in the development of SSc vasculopathy (23). Bleomycin-treated Fli1 haploinsufficient mice recapitulate most SSc features with dermal fibrosis, inflammation and vascular activation. They show increased dermal fibrosis, via the up-regulation of αvβ3 and αvβ5 integrins and activation of latent TGFβ as compared to wild-type control mice.

Vascular endothelial growth factor (VEGF) overexpression mice

SSc patients are characterized by the presence of high levels of VEGF in serum (24) and skin (25), albeit with defective angiogenesis. Recent data suggest a novel direct role for VEGF in fibrogenesis, providing a molecular link between vasculopathy and fibrosis (26). Double ($^{+/+}$) VEGF transgenic (tg) mice spontaneously develop skin fibrosis from 10 weeks onwards, with evidence of proliferative dermal vasculopathy. VEGF-tg mice have exaggerated fibrotic responses, both in the bleomycin model, modeling the early, inflammatory stages of the disease and the tight skin 1 (Tsk1) mouse, mimicking later non-inflammatory stages of SSc (26). It is not clear if upregulation of VEGF is an intrinsic part of disease or rather a compensatory consequence of vessel abnormalities in SSc with undesired negative effects on fibrogenesis.

Targeting the aggressor

Potential aggressors leading to vascular or epithelial damage as initiating events in SSc pathogenesis are still debated. Oxidative stress is put forward as a potential key player as SSc fibroblasts produce large amounts of reactive oxygen species (ROS) and this feature is associated with increased collagen synthesis (27). ROS production can also be linked to SSc vasculopathy as repetitive ischemia-reperfusion cycles typically generate superoxide anions (28). Some existing mouse models provide further support for these observations. Daily subcutaneous injections of specific ROS-generating solutions for 6 weeks in the shaved back skin of 6-week-old mice can reproduce both diffuse (dcSSc) and limited (lcSSc) cutaneous SSc (29).

Hypochlorous acid (HOCl)-induced fibrosis is another model of SSc in which repeated intradermal injections of HOCl mirror dcSSc with skin and lung fibrosis, renal arteriopathy and the presence of human disease associated antitopoisomerase-I antibodies (29). Skin involvement is characterized by increased dermal thickness, collagen content and fibroblast proliferation rate. In the lung thickened interalveolar septae, increased collagen content and T-cell infiltrates are observed. HOCL resulted in collagen accumulation in the renal interstitium and arteriopathy with intima/media thickening. Advanced oxidation protein products (AOPP), generated from DNA topoisomerase I (induced by HOCL) may link dermal and lung fibrosis in this model as serum AOPP concentrations were elevated in the dcSSc model and are present at increased levels in dcSSc patients. Experiments in severe combined immunodeficiency (SCID) mice show that a functional immune system is not required for the induction of skin fibrosis but is however required for full development of lung fibrosis. This model appears also amenable for therapeutic intervention. For instance treatment with simvastatin, a lipid-lowering drug that inhibits cholesterol synthesis prevented both dermal and pulmonary fibrosis (30). Interestingly, repeated intradermal injections of peroxynitrite ($ONOO^-$) resulted in dermal fibrosis and the presence of human disease associated anti-centromere antibodies (anti-CENP-B), however without evidence of systemic manifestations, therefore mirroring lcSSc (29).

Increased ROS levels are linked to damage and the initiation of fibrogenesis. However, when ROS levels are increased beyond a lethal, cytotoxic threshold, they could be used to selectively kill activated

fibroblasts and therefore applied therapeutically. The organo-tellurium-based catalyst 2,3-bis(phenyltellanyl)naphthoquinone ((PHTE)(2)NQ) was shown to prevent HOCl-induced murine systemic sclerosis with reduced levels of AOPP and antitopoisomerase-I antibodies, due to the selective pro-oxidative and cytotoxic effects of (PHTE)(2)NQ on hyperproliferative fibroblasts (31). Similarly, treatment with arsenic trioxide (As(2) O(3)) reduced dermal fibrosis in the HOCL model, abrogated vascular damage and inhibited autoantibody production through ROS-dependent killing of activated fibroblasts containing low levels of glutathione (32).

Autoimmunity and inflammatory changes

The role of autoimmunity as a primary or secondary event in the pathogenesis of SSc remains elusive. The presence of autoantibodies, different genetic associations with key molecules in the immune system and the recognition of inflammation in tissue biopsies are counterbalanced by the limited effects of broad immunosuppressive strategies. The current paradigm of SSc proposes that activation of the innate immune system influences adaptive immune responses and drives T-cell polarization following the lines of a classical immune reaction (33). Innate immunity associated surveillance cells such as dendritic (DC) and natural killer (NK) cells from SSc patients appear to be in a hyper-activated status, prone to stimulate Th2 skewed T-cell activation (34-36). Innate immune responses are mediated, in part, by Toll-like receptors (TLRs), which are evolutionarily conserved receptors for foreign pathogen-associated molecular patterns. Activation of TLR4 in SSc skin and lungs appears to increase collagen synthesis and enhance TGFβ sensitivity. Mice harboring a mutated TLR4 effectively have reduced skin fibrosis in the bleomycin-induced model (37). TLR4 knockout also attenuates skin and lung fibrosis in the bleomycin-induced model, and hypodermal fibrosis in the Tsk1 model ($TLR4^{-/-}$;$Tsk^{/+}$mice). In the $TLR4^{-/-}$ mice reduced inflammatory cytokine expression, abrogated IL-6 expression in fibroblasts, endothelial cells, and immune cells are recognized (38).

Aberrant activation of the adaptive immune system is evidenced by alterations in T- and B-cells. The presence of autoantibodies suggests abnormalities in the B-cell compartment (39). Microarray data on SSc skin reveal a clear B-cell signature (40) and SSc patients have a higher number of naïve B-cells with increased expression of activation markers (CD95) in memory B-cells. B-cell activating factor (BAFF) is expressed in the B-cell lineage and further activates B cells. In the bleomycin induced lung fibrosis model, BAFF levels are increased in the bronchoalveolar space. BAFF neutralization by a soluble receptor significantly attenuated pulmonary fibrosis and IL-1β levels (41). Belimumab is monoclonal antibody directed against BAFF. A Phase IIa study comparing belimumab/mycophenolate mofetil (MMF) to MMF alone is currently ongoing in dcSSc patients (NCT01670565).

In systemic sclerosis, lesional T-cells show a Th2 profile, with increased expression of profibrotic interleukin 4 (IL-4) (42). The specific reason for this preferred T cell profile is unknown. Of interest, the adhesion receptor DNAX accessory molecule-1 (DNAM-1) is present on a subset of T-cells and is overexpressed in SSc skin. Bleomycin-treated $Dnam1^{-/-}$ mice have significantly decreased numbers of T-cells in lesional skin, reduced TNF-α and IL-6 levels and appear protected from skin fibrosis development, indicating the regulatory role of DNAM-1 in T-cell activation and cytokine release. A DNAM-1 neutralizing mAb has potent antifibrotic properties (43).

The relationship between vascular changes and inflammation in the onset of SSc is unclear. Inflammatory cell migration is at least partially dependent on tissue exit of the cells through the vascular barrier. The interaction between leukocytes and the endothelium is mediated by the interaction between P-, E- and L-selectins and the leukocyte adhesion receptor P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 deficient mice were recently described as a model of progressive SSc with features of inflammation and autoimmunity, skin and lung fibrosis, microvasculopathy and internal organ involvement (44). From 3 months onwards, skin abnormalities are observed with superficial skin ulcers with minimal inflammatory infiltrates. With increasing age, the ulcers lesions grow and are accompanied by marked fibrosis and lipoatrophy in the underlying dermis. There is a significant reduction in the dermal blood vessel number. SPGL-1 deficiency results in increased activation state of skin macrophages, an increased number of DCs, an imbalance in Teff/Treg cells, increased Th1, Th2 and Th17 cells with production of IL-5, IFN-γ and IL-17 and heightened proinflammatory cytokine production (IL-6, IL-22, IL-13). Sera from PSGL-1 deficient mice recognize several autoantigens (anti-topoisomerase I, anti-Sm, anti-U1RNP, anti-Jo1, anti-SSA/Ro), which can be co-expressed within one individual and with increasing levels with age. The mice also have

renal abnormalities, which at young (1.5-3 months) age only occur in females and consist of perivascular infiltrates with sclerotic glomeruli and tubularization of Bowman's capsules with evidence of renal infarctisation. With advancing age (12-24 months) females and males are equally affected. The lungs show progressive interstitial cellularity and thickened interstitium, occurring from 1.5-3 months onwards. At the age of 12-24 months, 55-65% of mice have pulmonary pathology resembling NSIP, associated vasculopathy with media thickening. Despite the absence of this selectin-interacting molecule, cell migration does not seem dramatically impaired and its effects may be associated more with loss of tolerance and lack of regulatory T-cells due to loss of PSGL-1 – Syk signaling in dendritic cells (44).

Effector cells in fibrosis

Different cells types appear to contribute to the fibrotic outcome of SSc. A key cellular player in this process is the tissue-resident fibroblast. Increased resistance to apoptosis, transdifferentiation into myofibroblasts, excessive collagen production and reduced cell turnover characterize SSc fibroblasts. Myofibroblasts are activated fibroblasts, expressing the marker α smooth muscle antigen (αSMA). Representing an intermediate stage between fibroblast and smooth muscle phenotype, these cells combine features of both cell types and are critical for connective tissue contraction and remodeling. Besides fibroblasts and myofibroblasts, various other cell types have been suggested to play a role in SSc and contribute to the fibrotic process.

The specific role of the epithelial cells in the process of fibrosis is still subject of ongoing debate. Epithelial cells of different origins have been shown to have the capacity to undergo epithelial-mesenchymal transition (EMT), with loss of epithelial markers (e.g. E-cadherin) and gain of mesenchymal markers (collagen production, increased mobility) (45) but the in vivo relevance of EMT in pulmonary fibrotic pathology remains controversial (51-53). Other cells types involved may be endothelial cells, pericytes and fibrocytes. Endothelial-mesenchymal transition (EndoMT) has been documented but its role in Ssc is unclear (46). In early SSc, vascular pericytes (specialized mesenchymal cells, associated with small vessel walls) appear activated and express markers suggesting a transitional stage to myofibroblast transdifferentiation (47). Finally, bone marrow-derived fibroblast precursors, CD34+ fibrocytes, circulate in small numbers in peripheral blood and are thought to play a role in pathologic fibrotic conditions (48). Cell migration as contributing mechanism has also been suggested for Sox2-expressing skin progenitor cells that were demonstrated to play a role in bleomycin-induced scleroderma and are recruited to fibrotic lesions in a CTGF-dependent manner (49). As progression of skin fibrosis is characterized by loss of hypodermal adipose tissue, recent work has focused on intradermal adipocytes. Cell fate mapping studies using an adiponectin promoter-driven Cre recombinase transgenic construct, have provided evidence that dermal myofibroblasts in bleomycin-induced skin fibrosis can originate from adiponectin-positive intradermal progenitors. These progenitors were found in the lesional dermis, lost their adipocyte markers and gained mesenchymal markers, in a process of adipocyte-to-myofibroblast transition (50). These findings shed new light on the phenomenon of loss of adipose tissue, reorienting it from a mere symptom to a pathogenic event, providing an interesting new therapeutic target (51).

Signaling pathways in fibrosis

The behavior of the cellular compartment is likely modulated by the presence of numerous soluble mediators, such as TGFβ, CTGF, PDGF, monocyte chemotactic protein 1 (MCP-1), ET-1 and serotonin (5-hydroxytryptamin- 5HT), which are present at increased levels in SSc. TGFβ is considered the master regulator of fibrosis. Not only fibroblasts and myofibroblasts, but also platelets and innate (monocytes and macrophages) and adaptive (T-cells) immune cells can produce TGFβ (2). Other pathways associated with tissue fibrosis include bone morphogenetic protein signaling (BMP, member of the TGFβ superfamily), wingless (Wnt), Notch and hedgehog signaling (52-55). Not surprising, these are examples of typical morphogenic pathways that play a role extending from early developmental patterning to tissue development, differentiation and growth, but also homeostasis, physiological and pathological remodeling. As mentioned above, the current paradigm supports an initial environmental agent that, in a genetic susceptible individual, triggers vasculopathy and an immune response that will subsequently initiate repair mechanisms. As adequate confinement is lacking however, these processes result in fibrosis. In this concept, developmental pathways are key processes to study, and steering of these programs could offer the required confinement and limitation of initiated repair mechanisms, resulting in regeneration.

<u>Wnt signaling</u>

WNTs are lipid-modified glycoproteins that bind to Frizzled receptors. In association with low-density lipoprotein receptor-related protein-5 or -6 co-receptors, the canonical Wnt cascade with its key mediator β-catenin is activated (56). Recent data define a key pro-fibrotic role for the Wnt signaling pathway in the pathophysiology of systemic sclerosis skin (57) and lung fibrosis (58). Both experimental skin (59, 60) and lung fibrosis (61, 62) are largely Wnt-dependent. Modulators of the Wnt signaling pathway activity appear promising yet challenging therapeutic targets (63).

Wnt10b-transgenic mice show progressive dermal fibrosis and were proposed as a mouse model of systemic sclerosis (64). FABP4-Wnt10b-tg mice harbor the WNT-10b gene under the control of the fatty acid-binding protein (FABP) 4 promoter, resulting in ectopic WNT-10b expression in both white and brown adipose tissue and bone marrow. At 6 months of age, Wnt 10b-transgenic mice show spontaneous dermal fibrosis with loss of adipose tissue and reduced PPAR-γ expression. FABP4-driven ectopic expression of Wnt10b results in TGFβ-independent (no difference in pSMAD2, no effect of Alk5 inhibition) upregulation of collagen expression and deposition, leading to the replacement of hypodermal fat by fibrous tissue, resulting in increased dermal thickness with increased number of myofibroblasts and mast cells. From a therapeutic point of view, modulation of Wnt signaling has gained increasing attention and momentum. Inactivation of tankyrases 1 and 2 (TNKS-1 and TNKS-2) inhibits canonical Wnt signaling by preventing the proteasomal degradation of axin, resulting in the degradation of the key pathway mediator β-catenin. Tankyrase inactivation prevented both bleomycin-induced dermal thickening and adenoviral overexpression of TβRI-driven (AdTβR) skin fibrosis, without evidence for toxicity. Tankyrases might be candidates for targeted therapies in SSc (65). Two pharmaceutical inhibitors of downstream canonical Wnt signaling, PKF118-310 and ICG-001, demonstrated beneficial effects both in preventing and reversing bleomycin-induced dermal fibrosis (59). Furthermore, the Wnt antagonists DKK1 and SFRP1 are downregulated by promoter hypermethylation in SSc patients. Inhibition of DNA methyltransferases using 5-aza-2'-deoxycytidine resulted in normalization of the expression levels of both antagonists and effectively ameliorated experimental fibrosis. These data demonstrate that targeting epigenetic alterations can have translational implications (66). Reduction of both canonical and non-canonical Wnt ligand secretion by fibroblasts through inactivation of evenness interrupted (EVI), a multipass transmembrane protein localized in the Golgi and at the cell surface, reduces experimental fibrosis by combined inhibition of canonical and non-canonical Wnt signaling in the mouse models of bleomycin-induced and AdTβR -induced fibrosis (67). All these accumulating data highlight the critical role of Wnt signaling in the fibrotic process while at the same time identifying the need for translational validation of animal model data sets and providing support for further dissection of the Wnt cascades' roles in SSc.

TGFβ signaling

As TGFβ is a pleiotropic cytokine and growth factor involved in many different homeostatic and pathological processes, attention of the research community has further shifted towards key players in the associated signaling pathway not only to understand the fine details of fibrosis but also to eventually discover more specific targets for therapeutic intervention. For instance, transgenic mice overexpressing the TGFβ coreceptor CD109 in the epidermis, are protected from bleomycin-induced skin fibrosis, suggesting that CD109 inhibits TGFβ signaling, regulates dermal-epidermal interactions and should be considered as a potential molecular target for therapeutic interventions (68).

Soluble guanylate cyclase (sGC) catalyzes the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate. Riociguat, a stimulator of sGC, was recently approved as a novel, effective and well-tolerated drug for the treatment of pulmonary arterial hypertension (PAH) (69, 70). Pharmaceutical GC stimulation can inhibit TGFβ-induced fibroblast activation and ameliorate bleomycin-induced skin fibrosis (71). Recent work added strength to these findings and demonstrated that riociguat in doses of 1 and 3 mg/kg twice a day has potent dose-dependent anti-fibrotic properties in vitro and in vivo in 3 different mouse models of skin fibrosis (bleomycin-induced skin fibrosis, chronic graft-versus-host (cGVHD) model and Tsk1 mice). Riociguat also ameliorated fibrosis of the gastrointestinal tract in the cGVHD model. These findings position riociguat as an interesting therapeutic candidate for the treatment of SSc. A randomized, placebo-controlled phase II study with riociguat in patients with SSc is currently ongoing (NCT02283762) (72).

Nintedanib is a tyrosine kinase inhibitor, recently approved in the EU for the treatment of idiopathic pulmonary fibrosis (IPF). In vitro, nintedanib reversed the activated state of SSc fibroblasts. In vivo,

nintedanib had potent antifibrotic effects in the bleomycin-induced skin fibrosis model, the murine cGVHD model and in Tsk1 mice, providing strong experimental evidence for clinical trials with nintedanib in SSc (73). Tribbles homologue 3 (TRB3) is a pseudokinase that modifies the activation of various intracellular signaling pathways. Recently, TRB3 was shown to be a novel profibrotic mediator in SSc, downstream of TGFβ, with increased expression both in human SSc fibroblasts and mouse models of fibrosis. Knockdown of TRB3 reduced the profibrotic effects of TGF-β both in vitro and in vivo in the bleomycin- induced and $T\beta RI^{CA}$ mouse model (74).

Heat shock proteins (Hsps) are a family of molecular chaperones upregulated in response to cellular stress. Most Hsp recognize non-native proteins, preventing their irreversible aggregation. Furthermore, they modulate antigen processing and presentation and interact with different kinases and transcription factors. Hsp90 plays a central role in folding and conformational stabilization of TGFβ receptors (TβRI and TβRII) and Src kinases, intracellular mediators of the profibrotic TGFβ-effects. Hsp90 is upregulated in SSc skin and mouse models of skin fibrosis. Inhibition of Hsp90, accelerating ubiquitination and proteasomal degradation of TGFβR and Src, inhibited canonical TGFβ signaling and completely prevented the profibrotic effects of TGFβ both in vitro and in different in vivo models of fibrosis (bleomycin-induced dermal fibrosis, Tsk1 mice, $T\beta RI^{CA}$ mice) without indications of toxicity. Hsp90 inhibitors are currently in clinical trials for oncological indications and are possible interesting targets in SSc clinical research (75).

A short critical perspective on therapeutic targeting

The interplay between different signaling pathways as well as the distinct disease features in SSc remains incompletely understood. In recent years, multiple pathways have been targeted in experimental fibrosis models, often with promising and spectacular clinical results. Besides the accepted central role of TGFβ signaling, other developmental pathways such as Notch (53), hedgehog (55) and Wnt signaling (54) have been investigated and appear to play biologically relevant roles. Likewise, targeting other pathways e.g. the serotonin pathway (76), the cannabinoid system (77) and endothelin-dependent signaling (77) also successfully prevented and/or treated bleomycin-induced fibrosis. Inhibition of downstream kinase activity targeting Abelson (Abl) tyrosine kinase using imatinib (78), Janus kinase 2 (JAK2) (79) or Jun N-terminal kinase (80) resulted in inhibition of lung and/or skin fibrosis in the bleomycin-induced model. However, translation of these preclinical therapeutic targets to the clinical practice does not appear evident with both bosentan (81) and imatinib (82), targeting endothelin and Abl-kinase respectively, proven not to be effective in the treatment of lung fibrosis in large randomized placebo-controlled trials. Recent work has suggested that this discrepancy might be related to different activation levels of various drug targets in different mouse models and suggests that animal models for proof-of-concept studies should be selected based on a similar activation level and expression pattern of drug targets as in human SSc (83). Finally, interference with morphogen pathways carries the inherent risk of interference with stem cell regeneration, potentially complicating their use. Recently it was shown that combination therapies with low doses of Hedgehog/Wnt inhibitors or Hedgehog/Notch inhibitors demonstrated additive antifibrotic effects, were well tolerated and did not result in a reduction of intestinal stem cell numbers, providing a rationale to overcome dose-limiting toxicity of Hedgehog, Wnt and Notch signaling inhibition (84).

REFERENCES

1. Gabrielli A, Avvedimento EV, Krieg T. 2009. Scleroderma. *N Engl J Med* 360: 1989-2003
2. Wynn TA. 2008. Cellular and molecular mechanisms of fibrosis. *J Pathol* 214: 199-210
3. Ishikawa O, Ishikawa H. 1992. Macrophage infiltration in the skin of patients with systemic sclerosis. *J Rheumatol* 19: 1202-6
4. Kraling BM, Maul GG, Jimenez SA. 1995. Mononuclear cellular infiltrates in clinically involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. *Pathobiology* 63: 48-56
5. Liakouli V, Cipriani P, Marrelli A, Alvaro S, Ruscitti P, Giacomelli R. 2011. Angiogenic cytokines and growth factors in systemic sclerosis. *Autoimmun Rev* 10: 590-4
6. Eferl R, Hasselblatt P, Rath M, Popper H, Zenz R, Komnenovic V, Idarraga MH, Kenner L, Wagner EF. 2008. Development of pulmonary fibrosis through a pathway involving the transcription factor Fra-2/AP-1. *Proc Natl Acad Sci U S A* 105: 10525-30

7. Maurer B, Busch N, Jungel A, Pileckyte M, Gay RE, Michel BA, Schett G, Gay S, Distler J, Distler O. 2009. Transcription factor fos-related antigen-2 induces progressive peripheral vasculopathy in mice closely resembling human systemic sclerosis. *Circulation* 120: 2367-76
8. Maurer B, Reich N, Juengel A, Kriegsmann J, Gay RE, Schett G, Michel BA, Gay S, Distler JH, Distler O. 2012. Fra-2 transgenic mice as a novel model of pulmonary hypertension associated with systemic sclerosis. *Ann Rheum Dis*
9. Venalis P, Kumanovics G, Schulze-Koops H, Distler A, Dees C, Zerr P, Palumbo-Zerr K, Czirjak L, Mackevic Z, Lundberg IE, Distler O, Schett G, Distler JH. 2015. Cardiomyopathy in murine models of systemic sclerosis. *Arthritis Rheumatol* 67: 508-16
10. Kramer M, Dees C, Huang J, Schlottmann I, Palumbo-Zerr K, Zerr P, Gelse K, Beyer C, Distler A, Marquez VE, Distler O, Schett G, Distler JH. 2013. Inhibition of H3K27 histone trimethylation activates fibroblasts and induces fibrosis. *Ann Rheum Dis* 72: 614-20
11. Manetti M, Allanore Y, Revillod L, Fatini C, Guiducci S, Cuomo G, Bonino C, Riccieri V, Bazzichi L, Liakouli V, Cipriani P, Giacomelli R, Abbate R, Bombardieri S, Valesini G, Montecucco C, Valentini G, Ibba-Manneschi L, Matucci-Cerinic M. 2011. A genetic variation located in the promoter region of the UPAR (CD87) gene is associated with the vascular complications of systemic sclerosis. *Arthritis Rheum* 63: 247-56
12. Dewerchin M, Nuffelen AV, Wallays G, Bouche A, Moons L, Carmeliet P, Mulligan RC, Collen D. 1996. Generation and characterization of urokinase receptor-deficient mice. *J Clin Invest* 97: 870-8
13. Manetti M, Rosa I, Milia AF, Guiducci S, Carmeliet P, Ibba-Manneschi L, Matucci-Cerinic M. 2014. Inactivation of urokinase-type plasminogen activator receptor (uPAR) gene induces dermal and pulmonary fibrosis and peripheral microvasculopathy in mice: a new model of experimental scleroderma? *Ann Rheum Dis* 73: 1700-9
14. Ben-David Y, Giddens EB, Bernstein A. 1990. Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. *Proc Natl Acad Sci U S A* 87: 1332-6
15. Czuwara-Ladykowska J, Shirasaki F, Jackers P, Watson DK, Trojanowska M. 2001. Fli-1 inhibits collagen type I production in dermal fibroblasts via an Sp1-dependent pathway. *J Biol Chem* 276: 20839-48
16. Kubo M, Czuwara-Ladykowska J, Moussa O, Markiewicz M, Smith E, Silver RM, Jablonska S, Blaszczyk M, Watson DK, Trojanowska M. 2003. Persistent down-regulation of Fli1, a suppressor of collagen transcription, in fibrotic scleroderma skin. *Am J Pathol* 163: 571-81
17. Wang Y, Fan PS, Kahaleh B. 2006. Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis Rheum* 54: 2271-9
18. Asano Y, Czuwara J, Trojanowska M. 2007. Transforming growth factor-beta regulates DNA binding activity of transcription factor Fli1 by p300/CREB-binding protein-associated factor-dependent acetylation. *J Biol Chem* 282: 34672-83
19. Spyropoulos DD, Pharr PN, Lavenburg KR, Jackers P, Papas TS, Ogawa M, Watson DK. 2000. Hemorrhage, impaired hematopoiesis, and lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. *Mol Cell Biol* 20: 5643-52
20. Rao VN, Ohno T, Prasad DD, Bhattacharya G, Reddy ES. 1993. Analysis of the DNA-binding and transcriptional activation functions of human Fli-1 protein. *Oncogene* 8: 2167-73
21. Asano Y, Markiewicz M, Kubo M, Szalai G, Watson DK, Trojanowska M. 2009. Transcription factor Fli1 regulates collagen fibrillogenesis in mouse skin. *Mol Cell Biol* 29: 425-34
22. Nakerakanti SS, Kapanadze B, Yamasaki M, Markiewicz M, Trojanowska M. 2006. Fli1 and Ets1 have distinct roles in connective tissue growth factor/CCN2 gene regulation and induction of the profibrotic gene program. *J Biol Chem* 281: 25259-69
23. Asano Y, Stawski L, Hant F, Highland K, Silver R, Szalai G, Watson DK, Trojanowska M. 2010. Endothelial Fli1 deficiency impairs vascular homeostasis: a role in scleroderma vasculopathy. *Am J Pathol* 176: 1983-98
24. Allanore Y, Borderie D, Lemarechal H, Ekindjian OG, Kahan A. 2004. Nifedipine decreases sVCAM-1 concentrations and oxidative stress in systemic sclerosis but does not affect the concentrations of vascular endothelial growth factor or its soluble receptor 1. *Arthritis Res Ther* 6: R309-14

25. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, Michel BA, Gay RE, Muller-Ladner U, Matucci-Cerinic M, Plate KH, Gassmann M, Gay S. 2004. Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ Res* 95: 109-16
26. Maurer B, Distler A, Suliman YA, Gay RE, Michel BA, Gay S, Distler JH, Distler O. 2014. Vascular endothelial growth factor aggravates fibrosis and vasculopathy in experimental models of systemic sclerosis. *Ann Rheum Dis* 73: 1880-7
27. Sambo P, Jannino L, Candela M, Salvi A, Donini M, Dusi S, Luchetti MM, Gabrielli A. 1999. Monocytes of patients wiht systemic sclerosis (scleroderma spontaneously release in vitro increased amounts of superoxide anion. *J Invest Dermatol* 112: 78-84
28. Herrick AL, Matucci Cerinic M. 2001. The emerging problem of oxidative stress and the role of antioxidants in systemic sclerosis. *Clin Exp Rheumatol* 19: 4-8
29. Servettaz A, Goulvestre C, Kavian N, Nicco C, Guilpain P, Chereau C, Vuiblet V, Guillevin L, Mouthon L, Weill B, Batteux F. 2009. Selective oxidation of DNA topoisomerase 1 induces systemic sclerosis in the mouse. *J Immunol* 182: 5855-64
30. Bagnato G, Bitto A, Pizzino G, Irrera N, Sangari D, Cinquegrani M, Roberts WN, Matucci Cerinic M, Squadrito F, Altavilla D, Bagnato G, Saitta A. 2013. Simvastatin attenuates the development of pulmonary and cutaneous fibrosis in a murine model of systemic sclerosis. *Rheumatology (Oxford)* 52: 1377-86
31. Marut WK, Kavian N, Servettaz A, Nicco C, Ba LA, Doering M, Chereau C, Jacob C, Weill B, Batteux F. 2012. The organotelluride catalyst (PHTE)(2)NQ prevents HOCl-induced systemic sclerosis in mouse. *J Invest Dermatol* 132: 1125-32
32. Kavian N, Marut W, Servettaz A, Nicco C, Chereau C, Lemarechal H, Borderie D, Dupin N, Weill B, Batteux F. 2012. Reactive oxygen species-mediated killing of activated fibroblasts by arsenic trioxide ameliorates fibrosis in a murine model of systemic sclerosis. *Arthritis Rheum* 64: 3430-40
33. van Bon L, Cossu M, Radstake TR. 2011. An update on an immune system that goes awry in systemic sclerosis. *Curr Opin Rheumatol* 23: 505-10
34. Horikawa M, Hasegawa M, Komura K, Hayakawa I, Yanaba K, Matsushita T, Takehara K, Sato S. 2005. Abnormal natural killer cell function in systemic sclerosis: altered cytokine production and defective killing activity. *J Invest Dermatol* 125: 731-7
35. Sakkas LI, Xu B, Artlett CM, Lu S, Jimenez SA, Platsoucas CD. 2002. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. *J Immunol* 168: 3649-59
36. van Bon L, Popa C, Huijbens R, Vonk M, York M, Simms R, Hesselstrand R, Wuttge DM, Lafyatis R, Radstake TR. 2010. Distinct evolution of TLR-mediated dendritic cell cytokine secretion in patients with limited and diffuse cutaneous systemic sclerosis. *Ann Rheum Dis* 69: 1539-47
37. Bhattacharyya S, Kelley K, Melichian DS, Tamaki Z, Fang F, Su Y, Feng G, Pope RM, Budinger GR, Mutlu GM, Lafyatis R, Radstake T, Feghali-Bostwick C, Varga J. 2013. Toll-like receptor 4 signaling augments transforming growth factor-beta responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma. *Am J Pathol* 182: 192-205
38. Takahashi T, Asano Y, Ichimura Y, Toyama T, Taniguchi T, Noda S, Akamata K, Tada Y, Sugaya M, Kadono T, Sato S. 2015. Amelioration of tissue fibrosis by toll-like receptor 4 knockout in murine models of systemic sclerosis. *Arthritis Rheumatol* 67: 254-65
39. Daoussis D, Liossis SN, Yiannopoulos G, Andonopoulos AP. 2011. B-cell depletion therapy in systemic sclerosis: experimental rationale and update on clinical evidence. *Int J Rheumatol* 2011: 214013
40. Whitfield ML, Finlay DR, Murray JI, Troyanskaya OG, Chi JT, Pergamenschikov A, McCalmont TH, Brown PO, Botstein D, Connolly MK. 2003. Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A* 100: 12319-24
41. Francois A, Gombault A, Villeret B, Alsaleh G, Fanny M, Gasse P, Adam SM, Crestani B, Sibilia J, Schneider P, Bahram S, Quesniaux V, Ryffel B, Wachsmann D, Gottenberg JE, Couillin I. 2015. B cell activating factor is central to bleomycin- and IL-17-mediated experimental pulmonary fibrosis. *J Autoimmun* 56: 1-11
42. Mavalia C, Scaletti C, Romagnani P, Carossino AM, Pignone A, Emmi L, Pupilli C, Pizzolo G, Maggi E, Romagnani S. 1997. Type 2 helper T-cell predominance and high CD30 expression in systemic sclerosis. *Am J Pathol* 151: 1751-8

43. Avouac J, Elhai M, Tomcik M, Ruiz B, Friese M, Piedavent M, Colonna M, Bernhardt G, Kahan A, Chiocchia G, Distler JH, Allanore Y. 2013. Critical role of the adhesion receptor DNAX accessory molecule-1 (DNAM-1) in the development of inflammation-driven dermal fibrosis in a mouse model of systemic sclerosis. *Ann Rheum Dis* 72: 1089-98
44. Perez-Frias A, Gonzalez-Tajuelo R, Nunez-Andrade N, Tejedor R, Garcia-Blanco MJ, Vicente-Rabaneda E, Castaneda S, Gamallo C, Silvan J, Esteban-Villafruela A, Cubero-Rueda L, Garcia-Garcia C, Munoz-Calleja C, Garcia-Diez A, Urzainqui A. 2014. Development of an autoimmune syndrome affecting the skin and internal organs in P-selectin glycoprotein ligand 1 leukocyte receptor-deficient mice. *Arthritis Rheumatol* 66: 3178-89
45. Kalluri R, Neilson EG. 2003. Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112: 1776-84
46. Piera-Velazquez S, Li Z, Jimenez SA. 2011. Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. *Am J Pathol* 179: 1074-80
47. Rajkumar VS, Howell K, Csiszar K, Denton CP, Black CM, Abraham DJ. 2005. Shared expression of phenotypic markers in systemic sclerosis indicates a convergence of pericytes and fibroblasts to a myofibroblast lineage in fibrosis. *Arthritis Res Ther* 7: R1113-23
48. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. 1994. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1: 71-81
49. Liu S, Herault Y, Pavlovic G, Leask A. 2014. Skin progenitor cells contribute to bleomycin-induced skin fibrosis. *Arthritis Rheumatol* 66: 707-13
50. Marangoni RG, Korman BD, Wei J, Wood TA, Graham LV, Whitfield ML, Scherer PE, Tourtellotte WG, Varga J. 2015. Myofibroblasts in murine cutaneous fibrosis originate from adiponectin-positive intradermal progenitors. *Arthritis Rheumatol* 67: 1062-73
51. Bogatkevich GS. 2015. Editorial: fate of fat tissue adipocytes: do they transform into myofibroblasts in scleroderma? *Arthritis Rheumatol* 67: 860-1
52. Dees C, Tomcik M, Zerr P, Akhmetshina A, Horn A, Palumbo K, Beyer C, Zwerina J, Distler O, Schett G, Distler JH. 2011. Notch signalling regulates fibroblast activation and collagen release in systemic sclerosis. *Ann Rheum Dis* 70: 1304-10
53. Dees C, Zerr P, Tomcik M, Beyer C, Horn A, Akhmetshina A, Palumbo K, Reich N, Zwerina J, Sticherling M, Mattson MP, Distler O, Schett G, Distler JH. 2011. Inhibition of Notch signaling prevents experimental fibrosis and induces regression of established fibrosis. *Arthritis Rheum* 63: 1396-404
54. Akhmetshina A, Palumbo K, Dees C, Bergmann C, Venalis P, Zerr P, Horn A, Kireva T, Beyer C, Zwerina J, Schneider H, Sadowski A, Riener MO, MacDougald OA, Distler O, Schett G, Distler JH. 2012. Activation of canonical Wnt signalling is required for TGF-beta-mediated fibrosis. *Nat Commun* 3: 735
55. Horn A, Kireva T, Palumbo-Zerr K, Dees C, Tomcik M, Cordazzo C, Zerr P, Akhmetshina A, Ruat M, Distler O, Beyer C, Schett G, Distler JH. 2012. Inhibition of hedgehog signalling prevents experimental fibrosis and induces regression of established fibrosis. *Ann Rheum Dis*
56. Clevers H, Nusse R. 2012. Wnt/beta-Catenin Signaling and Disease. *Cell* 149: 1192-205
57. Beyer C, Schramm A, Akhmetshina A, Dees C, Kireva T, Gelse K, Sonnylal S, de Crombrugghe B, Taketo MM, Distler O, Schett G, Distler JH. 2012. beta-catenin is a central mediator of pro-fibrotic Wnt signaling in systemic sclerosis. *Ann Rheum Dis* 71: 761-7
58. Lam AP, Flozak AS, Russell S, Wei J, Jain M, Mutlu GM, Budinger GR, Feghali-Bostwick CA, Varga J, Gottardi CJ. 2011. Nuclear beta-catenin is increased in systemic sclerosis pulmonary fibrosis and promotes lung fibroblast migration and proliferation. *Am J Respir Cell Mol Biol* 45: 915-22
59. Beyer C, Reichert H, Akan H, Mallano T, Schramm A, Dees C, Palumbo-Zerr K, Lin NY, Distler A, Gelse K, Varga J, Distler O, Schett G, Distler JH. 2013. Blockade of canonical Wnt signalling ameliorates experimental dermal fibrosis. *Ann Rheum Dis* 72: 1255-8
60. Bergmann C, Akhmetshina A, Dees C, Palumbo K, Zerr P, Beyer C, Zwerina J, Distler O, Schett G, Distler JH. 2011. Inhibition of glycogen synthase kinase 3beta induces dermal fibrosis by activation of the canonical Wnt pathway. *Ann Rheum Dis* 70: 2191-8
61. Henderson WR, Jr., Chi EY, Ye X, Nguyen C, Tien YT, Zhou B, Borok Z, Knight DA, Kahn M. 2010. Inhibition of Wnt/beta-catenin/CREB binding protein (CBP) signaling reverses pulmonary fibrosis. *Proc Natl Acad Sci U S A* 107: 14309-14

62. Beyer C, Dees C, Distler JH. 2013. Morphogen pathways as molecular targets for the treatment of fibrosis in systemic sclerosis. *Arch Dermatol Res* 305: 1-8
63. De Langhe E, Aznar-Lopez C, De Vooght V, Vanoirbeek JA, Luyten FP, Lories RJ. 2014. Secreted frizzled related proteins inhibit fibrosis in vitro but appear redundant in vivo. *Fibrogenesis Tissue Repair* 7: 14
64. Wei J, Melichian D, Komura K, Hinchcliff M, Lam AP, Lafyatis R, Gottardi CJ, MacDougald OA, Varga J. 2011. Canonical Wnt signaling induces skin fibrosis and subcutaneous lipoatrophy: a novel mouse model for scleroderma? *Arthritis Rheum* 63: 1707-17
65. Distler A, Deloch L, Huang J, Dees C, Lin NY, Palumbo-Zerr K, Beyer C, Weidemann A, Distler O, Schett G, Distler JH. 2013. Inactivation of tankyrases reduces experimental fibrosis by inhibiting canonical Wnt signalling. *Ann Rheum Dis* 72: 1575-80
66. Dees C, Schlottmann I, Funke R, Distler A, Palumbo-Zerr K, Zerr P, Lin NY, Beyer C, Distler O, Schett G, Distler JH. 2014. The Wnt antagonists DKK1 and SFRP1 are downregulated by promoter hypermethylation in systemic sclerosis. *Ann Rheum Dis* 73: 1232-9
67. Distler A, Ziemer C, Beyer C, Lin NY, Chen CW, Palumbo-Zerr K, Dees C, Weidemann A, Distler O, Schett G, Distler JH. 2014. Inactivation of evenness interrupted (EVI) reduces experimental fibrosis by combined inhibition of canonical and non-canonical Wnt signalling. *Ann Rheum Dis* 73: 624-7
68. Vorstenbosch J, Al-Ajmi H, Winocour S, Trzeciak A, Lessard L, Philip A. 2013. CD109 overexpression ameliorates skin fibrosis in a mouse model of bleomycin-induced scleroderma. *Arthritis Rheum* 65: 1378-83
69. Rubin LJ, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, Keogh A, Langleben D, Fritsch A, Menezes F, Davie N, Ghofrani HA. 2015. Riociguat for the treatment of pulmonary arterial hypertension: a long-term extension study (PATENT-2). *Eur Respir J* 45: 1303-13
70. Ghofrani HA, Galie N, Grimminger F, Grunig E, Humbert M, Jing ZC, Keogh AM, Langleben D, Kilama MO, Fritsch A, Neuser D, Rubin LJ, Group P-S. 2013. Riociguat for the treatment of pulmonary arterial hypertension. *N Engl J Med* 369: 330-40
71. Beyer C, Zenzmaier C, Palumbo-Zerr K, Mancuso R, Distler A, Dees C, Zerr P, Huang J, Maier C, Pachowsky ML, Friebe A, Sandner P, Distler O, Schett G, Berger P, Distler JH. 2014. Stimulation of the soluble guanylate cyclase (sGC) inhibits fibrosis by blocking non-canonical TGFbeta signalling. *Ann Rheum Dis*
72. Dees C, Beyer C, Distler A, Soare A, Zhang Y, Palumbo-Zerr K, Distler O, Schett G, Sandner P, Distler JH. 2015. Stimulators of soluble guanylate cyclase (sGC) inhibit experimental skin fibrosis of different aetiologies. *Ann Rheum Dis*
73. Huang J, Beyer C, Palumbo-Zerr K, Zhang Y, Ramming A, Distler A, Gelse K, Distler O, Schett G, Wollin L, Distler JH. 2015. Nintedanib inhibits fibroblast activation and ameliorates fibrosis in preclinical models of systemic sclerosis. *Ann Rheum Dis*
74. Tomcik M, Palumbo-Zerr K, Zerr P, Sumova B, Avouac J, Dees C, Distler A, Becvar R, Distler O, Schett G, Senolt L, Distler JH. 2015. Tribbles homologue 3 stimulates canonical TGF-beta signalling to regulate fibroblast activation and tissue fibrosis. *Ann Rheum Dis*
75. Tomcik M, Zerr P, Pitkowski J, Palumbo-Zerr K, Avouac J, Distler O, Becvar R, Senolt L, Schett G, Distler JH. 2014. Heat shock protein 90 (Hsp90) inhibition targets canonical TGF-beta signalling to prevent fibrosis. *Ann Rheum Dis* 73: 1215-22
76. Dees C, Akhmetshina A, Zerr P, Reich N, Palumbo K, Horn A, Jungel A, Beyer C, Kronke G, Zwerina J, Reiter R, Alenina N, Maroteaux L, Gay S, Schett G, Distler O, Distler JH. 2011. Platelet-derived serotonin links vascular disease and tissue fibrosis. *J Exp Med* 208: 961-72
77. Marquart S, Zerr P, Akhmetshina A, Palumbo K, Reich N, Tomcik M, Horn A, Dees C, Engel M, Zwerina J, Distler O, Schett G, Distler JH. 2010. Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. *Arthritis Rheum* 62: 3467-76
78. Daniels CE, Wilkes MC, Edens M, Kottom TJ, Murphy SJ, Limper AH, Leof EB. 2004. Imatinib mesylate inhibits the profibrogenic activity of TGF-beta and prevents bleomycin-mediated lung fibrosis. *J Clin Invest* 114: 1308-16
79. Dees C, Tomcik M, Palumbo-Zerr K, Distler A, Beyer C, Lang V, Horn A, Zerr P, Zwerina J, Gelse K, Distler O, Schett G, Distler JH. 2012. JAK-2 as a novel mediator of the profibrotic effects of transforming growth factor beta in systemic sclerosis. *Arthritis Rheum* 64: 3006-15

80. Reich N, Tomcik M, Zerr P, Lang V, Dees C, Avouac J, Palumbo K, Horn A, Akhmetshina A, Beyer C, Xie W, Bennett BL, Distler O, Schett G, Distler JH. 2012. Jun N-terminal kinase as a potential molecular target for prevention and treatment of dermal fibrosis. *Ann Rheum Dis* 71: 737-45
81. Seibold JR, Denton CP, Furst DE, Guillevin L, Rubin LJ, Wells A, Matucci Cerinic M, Riemekasten G, Emery P, Chadha-Boreham H, Charef P, Roux S, Black CM. 2010. Randomized, prospective, placebo-controlled trial of bosentan in interstitial lung disease secondary to systemic sclerosis. *Arthritis Rheum* 62: 2101-8
82. Daniels CE, Lasky JA, Limper AH, Mieras K, Gabor E, Schroeder DR. 2010. Imatinib treatment for idiopathic pulmonary fibrosis: Randomized placebo-controlled trial results. *Am J Respir Crit Care Med* 181: 604-10
83. Maurer B, Distler A, Dees C, Khan K, Denton CP, Abraham D, Gay RE, Michel BA, Gay S, Hw Distler J, Distler O. 2013. Levels of target activation predict antifibrotic responses to tyrosine kinase inhibitors. *Ann Rheum Dis* 72: 2039-46
84. Distler A, Lang V, Del Vecchio T, Huang J, Zhang Y, Beyer C, Lin NY, Palumbo-Zerr K, Distler O, Schett G, Distler JH. 2014. Combined inhibition of morphogen pathways demonstrates additive antifibrotic effects and improved tolerability. *Ann Rheum Dis* 73: 1264-8